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Low larval densities in northern populations reinforce range expansion by a Mediterranean damselfly

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SUMMARY

1. Contemporary climate change triggers a poleward range shift in many species. A growing number of studies document evolutionary changes in traits accelerating range expansion (such as growth rate and dispersal-related traits). In contrast, the direct impact of decreasing conspecific densities towards the very edge of the expansion front has been neglected. Density effects may, however, have a profound direct impact on traits involved in range expansion and influence range dynamics.

2. In this study, we contrast the effects of high conspecific larval density typical for established populations and low larval density typical for newly founded populations at the edge of the expansion front on a set of larval traits that may affect the range dynamics in the poleward moving damselfly *Coenagrion scitulum*. We therefore ran an outdoor mesocosm experiment with a low- and high-density treatment close to the species' northern expansion front. Density effects on survival, growth rate and body size are scored both during the pre-winter growth period and during the subsequent winter period. Additionally, foraging activity was scored at the end of the pre-winter period and body condition [size-corrected body mass, fat content and activity of phenoloxidase (PO)] was scored at the end of the winter period.

3. The low-density treatment had strong direct positive effects on survival, growth rate and body size of larvae before winter indicating relaxed competition. Lower foraging activity at the low-density treatment indicated higher food availability at low conspecific densities. Interestingly, the initial density treatment had stronger effect than densities experienced at the time of quantification on survival during the pre-freezing winter period and body condition estimates at the end of the experiment, indicating also delayed effects of the initial density treatment. Survival throughout a freezing period indicated extreme winter conditions are not likely a limiting factor in the range expansion of this Mediterranean species.

4. The increased survival and individual growth rates (through causing shifts in voltinism) at low conspecific density will translate in increased population growth rates. Furthermore, nutritional advantages at low conspecific density may increase investment in dispersal ability. Together, these direct and delayed density-dependent effects that gradually increase towards the expansion front are expected to accelerate range expansion.

Keywords: global warming, intraspecific competition, odonata, range expansion, winter survival

Introduction

Triggered by contemporary climate change, ranges of many species are shifting poleward to previously unoccupied, but now suitable habitats (Hickling *et al.*, 2006; Chen *et al.*, 2011). Individuals at the expansion front experience different selective forces than those in established populations which drives the evolution of phenotypic traits during range expansion (Excoffier & Ray, 2008; Phillips, Brown & Shine, 2010a; Hill, Griffiths &

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Thomas, 2011). This is the case even when a species is perfectly tracking its moving climate niche. First, conspecific densities are initially lower at newly colonised patches, thereby favouring genotypes that are able to produce a larger number of generations in a given amount of time (Phillips, 2009; Phillips *et al*., 2010a). Second, only the best dispersers make it to the advancing expansion front each year; combined with fitness advantages for dispersers at the initial low population density at the expansion front, this can lead to strong selection for increased dispersal ability at the expansion front (Hughes, Dytham & Hill, 2007; Phillips, Brown & Shine, 2010b).

While evolutionary changes in traits accelerating range expansion (such as growth rate and dispersal-related traits) are well studied, the direct impact of differences in population density across the expansion gradient on these traits has been neglected. Newly founded range edge populations are characterised by low population densities and relaxed conspecific competition as patches at the expansion front are typically colonised by a small number of individuals (Burton & Travis, 2008). Theoretical models have shown an important impact of density regulation on the ability of tracking a shifting climate and local adaptation at the expansion front (Best *et al*., 2007; Filin, Holt & Barfield, 2008). Density effects may have a profound direct impact on traits involved in range expansion and therefore influence range dynamics. Low population densities reduce intraspecific competition leading to an increased food intake (McPeek & Crowley, 1987), resulting in increased survival and growth rates (Banks & Thompson, 1987; Anholt, 1990a). This has been shown to positively affect body condition and increase the number of generations per year (voltinism, Corbet, Suhling & Soendgerath, 2006). Additionally, climate conditions at northern range edges are often stressful due to shorter growth seasons and/or harsher winter conditions (Hardie & Hutchings, 2010) which potentially increase the negative impact of larval crowding at the northern expansion front.

The low conspecific densities at the range front are transient and population densities may increase fast after colonisation resulting in a steep gradient of low conspecific densities at the expansion front grading back to near-equilibrium densities behind the front (Phillips, 2009). Understanding the fine-scaled density-related processes that operate during range expansion is crucial to predict the fate of newly founded range front populations and the rate of future range expansion. The potential rate of evolution during range expansion increases with the number of iterated founder events (McInerny *et al*., 2009). Furthermore, costs of dispersal select for lower dispersal rates through which evolutionary changes induced by range expansion may vanish fast after population establishment (Simmons & Thomas, 2004). If populations at the range edge remain connected with established populations, the immigration of these alleles advantageous in established populations can swamp the alleles advantageous in edge populations (Lenormand, 2002; Bridle & Vines, 2007), thus impeding evolutionary trajectories at the moving range front.

In this study, we contrast the effects of conspecific larval densities typical for established populations (high larval density) and newly founded populations at the expansion front (low larval density) on a set of larval traits that may affect the range dynamics in the poleward moving damselfly *Coenagrion scitulum*. We therefore studied the density effects on survival, growth rate and body condition both during the favourable thermal growth conditions in the pre-winter growth period and during the harsh conditions in the winter period in an outdoor mesocosm experiment performed close to the species' northern expansion front. Simulations based on geographic variation in water temperature and thermal sensitivity of the species' larval growth rate indicate that the latitude of current expansion front is situated at a transition zone from an univoltine to a semivoltine life cycle (Nilsson-Örtman *et al*., 2012). This indicates that any density-induced reduction of larval growth rate at the latitude of the expansion front may cause a voltinism shift, and therefore, a doubling of the generation time. This will inevitably decline effective adult population size through higher competition and cannibalism by older age cohorts (Crowley *et al*., 1987; Anholt, 1994; Purse & Thompson, 2002) and increased predation risk during the extended larval period (Berger, Walters & Gotthard, 2006).

Methods

Study system and collection

Coenagrion scitulum (Coenagrionidae) is a Mediterranean damselfly preferring small ponds with rich aquatic vegetation (Dijkstra, 2006). Up to the 1990s, the northern range limit was situated at the latitude of Paris, France, after which a northeastward range expansion has occurred (Wasscher & Goudsmits, 2010). In 2010, the northernmost limit of the expanding range margin was situated in the southern parts of the Netherlands, and the northeastern limit in Western Germany (Swaegers *et al*., 2013). During the range expansion of *C. scitulum*, a

fast increase in population density is expected after the colonisation of patches at the expansion front through the capacity of *C. scitulum* to produce large egg clutches (Therry *et al*., 2014c) and by the observation of high population densities in established edge populations within 5 years after colonisation (Therry *et al*., 2014d). Accordingly, high-density populations and low-density populations are in close proximity across the expansion gradient in this study system. Evolution towards a faster life history, a higher investment in flight-related traits (Therry *et al*., 2014a,b) and selection for a genetic marker associated with increased flight performance (Swaegers *et al*., 2015) has been documented at the expansion front of *C. scitulum*. We studied three populations in Northwestern Europe (La ville-aux-bois, France; Merlimont, France; Cottessen, The Netherlands) which were located >200 km from each other (Fig. 1). The studied populations were chosen relatively close to the expansion front which enhances the relevance of our experiment as such populations are likely founder populations of further range expansion.

Paired females were caught during June-July 2011 and allowed to oviposit in plastic jars on wet filter paper, resulting in 12 egg clutches from each population. Eggs were transported to the laboratory in Belgium and the eggs and larvae were kept at 22 °C and a photoperiod of L : D 14 : 10 h until entered in the outdoor experiments. Directly after hatching, larvae were kept in batches of 15 in rectangular plastic containers (14.5 × 10 × 26 cm) and fed twice a day *ad libitum* with freshly hatched *Artemia* nauplii.

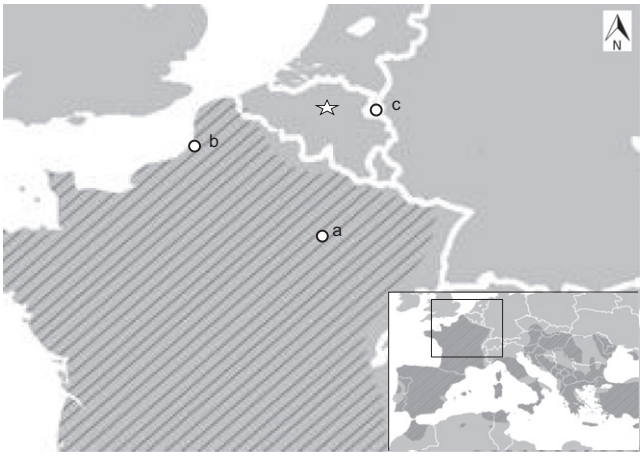

Fig. 1 Locations of the *Coenagrion scitulum* study populations and the northwestern part of the species' range at 2005 (shaded area based on Dijkstra, 2006; Boudot, 2013, unpubl. data). Circles represent the populations (a: La Ville-Aux-Bois, France; b: Merlimont, France; c: Cottessen, The Netherlands) and the open asterisk represents the location of the outdoor mesocosm study.

General experimental procedure

The density experiment was run in outdoor cylindrical plastic mesocosms (diameter: 22.5 cm, height: 26 cm) at a field site in Heverlee, Belgium (Fig. 1). Low densities started with groups of 10 larvae (ca. 252 larvae/m²), high densities with groups of 50 larvae (ca. 1258 larvae/m²) per mesocosm. The high-density treatment reflects field densities of early instar *Coenagrion* larvae at carrying density in established populations in suitable habitats (Corbet, 1999). Each treatment was replicated five times at the population level, resulting in a total of 30 mesocosms. The experiment was run over two consecutive periods, a pre-winter and a winter period, with the latter further divided in a pre-freezing and a freezing winter subperiod (Fig. 2). The pre-winter period constitutes a period of fast growth for *Coenagrion* damselfly larvae (Nilsson-Örtman *et al*., 2013), while during the winter period the larvae enter a diapause (Corbet, 1999). Performance of larvae during both periods was quantified by scoring survival and growth rates. At the end of

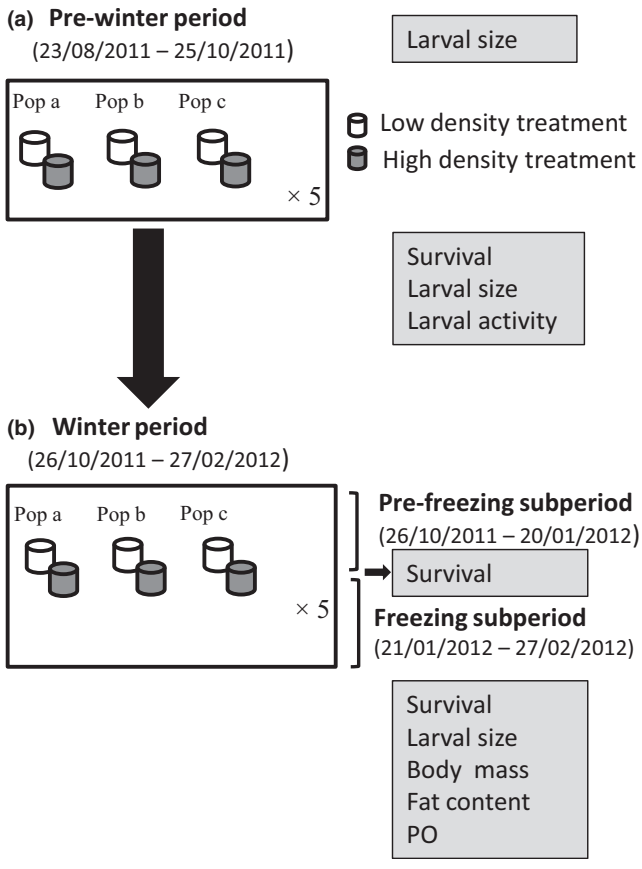

Fig. 2 Experimental set-up and measured response variables (grey shaded boxes) after the pre-winter (a) and winter period (b). The experimental set-up is described in the methods.

the pre-winter period, we scored larval foraging activity which provides information about food availability in the mesocosms as odonate larvae increase activity under limited food conditions (Werner & Anholt, 1993). At the end of the winter period, we estimated body condition by quantifying size-corrected body mass, fat content and the activity of the enzyme phenoloxidase (PO), a key component of insect immune function (González-Santoyo & Córdoba-Aguilar, 2012). The experimental set-up is described in detail below separately for the pre-winter and winter periods.

Pre-winter period

One week prior to the start of the outdoor experiment, 30 mesocosms were filled with a 5 L mixture of 50% filtered pond water and 50% tap water. Fresh grass was added to provide structure for the larvae and to stimulate growth of protists. Additionally, each mesocosm was inoculated with a culture of *Daphnia pulex*. Mesocosms were covered with mosquito netting to prevent colonisation by other species. Head width of a subset of larvae (24 larvae from each population) was measured using a binocular linked to an image analyser, before the initiation of the experiment. The experiment was initiated on 23 August 2011 by transporting the larvae from the laboratory to the outdoor mesocosms and the pre-winter period stopped 63 days later on 25 October 2011 when water temperature had dropped to 10 °C. This temperature is 2 °C higher than the temperature threshold where *Coenagrion* larvae stopped growing and enter diapause (Corbet, 1999). Collecting the larvae at 10 °C ensured that none of the larvae had entered diapause; this prevented complications when scoring foraging activity.

At the end of the pre-winter period, larvae were counted per mesocosm to estimate pre-winter survival. Six randomly chosen larvae (less if the number of survived larvae in the mesocosm was lower than six) of each mesocosm were transported to the laboratory for quantification of head width and foraging activity. Larval head width is an often used proxy for larval size in Odonata larvae (e.g. Brodin & Johansson, 2004; Mikolajewski *et al.*, 2005) and was measured using a binocular linked to an image analyser. Larval growth rate across the pre-winter period was calculated per mesocosm as [ln(mean final larval head width) − ln(mean initial head width)]/63 days.

Larval foraging activity was quantified as the number of *Artemia* nauplii each larva consumed during 30 min (Janssens & Stoks, 2013). Foraging trials were run individually in white cups (diameter: 6.5 cm, height: 9 cm) filled with 100 mL dechlorinated tap water. Cups with larvae were first kept for 12 h at 15 °C. This temperature was 5 °C warmer than the water temperature of the outdoor mesocosms at the end of the pre-winter period, but mimics the temperature during most part of the outdoor pre-winter experiment. Each foraging trial was initiated by adding 20 freshly hatched *Artemia* nauplii to the cups. After 30 minutes, the trial was ended by removing the damselfly larva and the remaining *Artemia* nauplii were counted. Afterwards, larvae were directly transported back to the experimental field.

Winter period

In the winter period, we tested if larvae reared at high and low densities cope differentially with winter conditions. At the start of the winter period, all larvae of each mesocosm were transferred to 5 L cylindrical plastic cages (diameter: 20 cm, height: 22 cm). Each cage was kept floating in the middle of a large cylindrical 210 L tank (diameter bottom: 52 cm, diameter top: 66 cm, height: 80 cm) using polystyrene floaters. The large water volume of the tank buffered short-term freezing of cages when environmental temperature balanced around freezing point. Each cage had two 5 × 5 cm holes on opposite sides covered by 200-μm mesh to allow water exchange between the cage and the 210 L tank to prevent nutrient accumulation and anoxic conditions in the cages. The number of *Daphnia* prey was visually checked twice per week throughout the winter period and prey density was kept at equal levels across mesocosms by redistributing *Daphnia* across mesocosms.

The winter period was divided in a pre-freezing (26 October 2011 until 20 January 2012) and a freezing period (21 January 2012 until 27 February 2012). Data loggers measuring the water temperature every 6 hours were placed at the bottom of two randomly chosen cages. The mean temperature at the bottom in the cages was 6.0 °C and 3.1 °C during the pre-freezing and freezing winter subperiods respectively. During the freezing winter subperiod, the bottom temperature was below 0 °C for 14 days, indicating that the cages were totally frozen and the larvae were fully encapsulated in the ice during this subperiod.

Survival of larvae was scored during both winter subperiods. At the end of the winter period, final size, body mass and size-corrected body mass of the larvae were scored. Afterwards, larvae were flash-frozen and stored at −80 °C until quantification of physiological variables (fat reserves and PO activity).

Quantification of fat reserves and investment in immune function

We quantified two physiological variables associated with body condition, fat content and activity of phenoloxidase (PO) of the larvae sampled after the winter period. PO is a key component of insect immune function and catalyses melanin formation, used to melanise foreign objects and is involved in the production of quinine intermediates, cytotoxic superoxide anions and hydroxyl radicals that participate in pathogen destruction (Nappi & Christensen, 2005). Without immune challenge, PO activity reflects body condition (González-Santoyo & Córdoba-Aguilar, 2012). Fat is the main long-term energy storage molecule of insects (Klowden, 2002).

Larvae were homogenised in PBS buffer (50 mM, pH 7.4; ratio: 20 μL PBS buffer per mg wet body mass) followed by a centrifugation for 8 min (4 °C, 13 000 rpm) to remove the cell membranes and the exoskeleton. After centrifugation, the supernatant was pipetted to 1.5 mL eppendorf tubes. To quantify PO activity, 2.5 μL of the supernatant, 2.5 μL milli-Q water and 5 μL of α-chymotrypsine (0.20 M) was pipetted into a well of a 384-well microplate (Greiner 384 Flatblack). α-chymotrypsin catalyses the transition of the pro-enzyme proPO to PO. After 5 min, 30 μl L-dopa (0.010 M) substrate was added. We monitored the transition from L-dopa to dopachrome by recording the increase in absorbance at 490 nm in a spectrophotometer at 30 °C. The reaction was allowed to proceed for 30 min and readings were taken every 20 s. Enzyme activity was measured as the slope during the linear phase of the reaction where the PO catalyses the transition of L-dopa to dopachrome.

To quantify the total fat content of larvae, 8 μL supernatant and 56 μL H_2SO_4 (100%) was mixed in a well of a 384-well microplate. This mixture was incubated at 200 °C for 20 min and then cooled down at room temperature. Afterwards, 64 μL milli-Q water was added and mixed. A quantity of 30 μl of the mixture was pipetted into a well of a 384-well microplate and the absorbance of the samples was measured after 20 min at 340 mm. Total fat content was expressed as microgram fat/mg wet mass. Quantifications of PO activity and fat content were executed in duplicate and the mean per larva was used for the statistical analyses.

Statistical analyses

All data were analysed using general mixed models in SAS v. 9.3 with population and mesocosm nested in population as random factors. This way, we took into account that sets of larvae had been reared together in certain mesocosms and thereby avoided pseudoreplication. The effect of the density treatment (low versus high) on the log-transformed number of larvae alive at the end of the three different (sub)periods (pre-winter period, pre-freezing subperiod and freezing subperiod) was analysed with separate ANOVAs per period. Similarly, the effect of the density treatment on larval survival during each (sub)period was analysed using separate ANOVAs with binomial error structure. At the end of the pre-winter period, larval densities were still higher at the high-density treatment than at the low-density treatment. Yet, larval densities did no longer differ between the two density treatments at the end of the pre-freezing winter period and the end of the winter experiment (see Results). We therefore additionally tested in separate analyses the effect of the log-transformed initial densities at the start of the considered winter subperiod on survival during the pre-freezing and freezing winter subperiods.

The effect of the density treatment on head width at the end of the pre-winter and winter periods and on growth rate during the pre-winter and winter periods were analysed using ANOVAs. Measurements of head width after the winter period were obtained from both larvae that survived the freezing period and larvae that were frozen to death. To test if larvae that died during the freezing period differed in size from these that survived the freezing period, survival status (alive versus death) was added as an independent factor to the analyses of head width.

The effect of the density treatment on log-transformed foraging activity measured after the pre-winter period was tested using an ANCOVA. The larval head width was added as a covariate when testing for larval foraging activity. The effect of the density treatment on body mass, fat content and PO activity of larvae after the winter period were analysed with ANCOVAs. To correct for the effects of the final density which was no longer dependent on the initial density treatment, we added to each analysis the log-transformed final density as covariate. The analysis of body mass was performed twice, once without and once with size correction (the addition of head width as covariate) in order to obtain a size-independent measure for condition (Braune & Rolff, 2001).

Results

The number of larvae present in the mesocosms at the end of the pre-winter period was still higher at the high-density than at the low-density treatments. Yet, the

number of larvae did no longer differ between both density treatments at the end of the pre-freezing and the freezing winter subperiods (Table 1a, Fig. 3a). Larval survival during the pre-winter period and the pre-freezing winter subperiod was higher at the low-density treatment, while the density treatment did not influence survival during the freezing winter subperiod (Table 1b, Fig. 3b). In contrast, neither during the pre-freezing winter subperiod nor during the freezing winter subperiod was survival dependent on the initial larval density at the start of the respective subperiod (Table 1c).

The head width of larvae was higher at the low-density treatment at both the end of the pre-winter period and at the end of the winter period (Table 2a, Fig. 4a). Larvae were growing faster at the low-density treatment during the pre-winter period. In contrast, larvae hardly

Table 1 ANOVAs testing for the effect of the density treatment (low versus high) on (a) the number of larvae alive of *Coenagrion scitulum* at the end of each (sub)period (pre-winter period, pre-freezing winter subperiod and freezing winter subperiod) and (b) survival during the three (sub)periods. (c) ANCOVAs testing for the effect of initial density (larval density at the start of that period) on survival during both winter periods (pre-freezing and freezing period).

	Pre-winter period			Pre-freezing winter subperiod			Freezing winter subperiod		
	d.f.	*F*	*P*	d.f.	*F*	*P*	d.f.	*F*	*P*
(a) Number of larvae alive at the end									
Density treatment	1,30	12.49	0.001	1,30	1.92	0.176	1,30	2.16	0.152
(b) Survival during period									
Density treatment	1,870	27.59	<0.001	1,219	8.22	0.005	1,157	0.59	0.443
(c) Survival during period									
Initial density				1,218	0.84	0.360	1,156	0.04	0.851

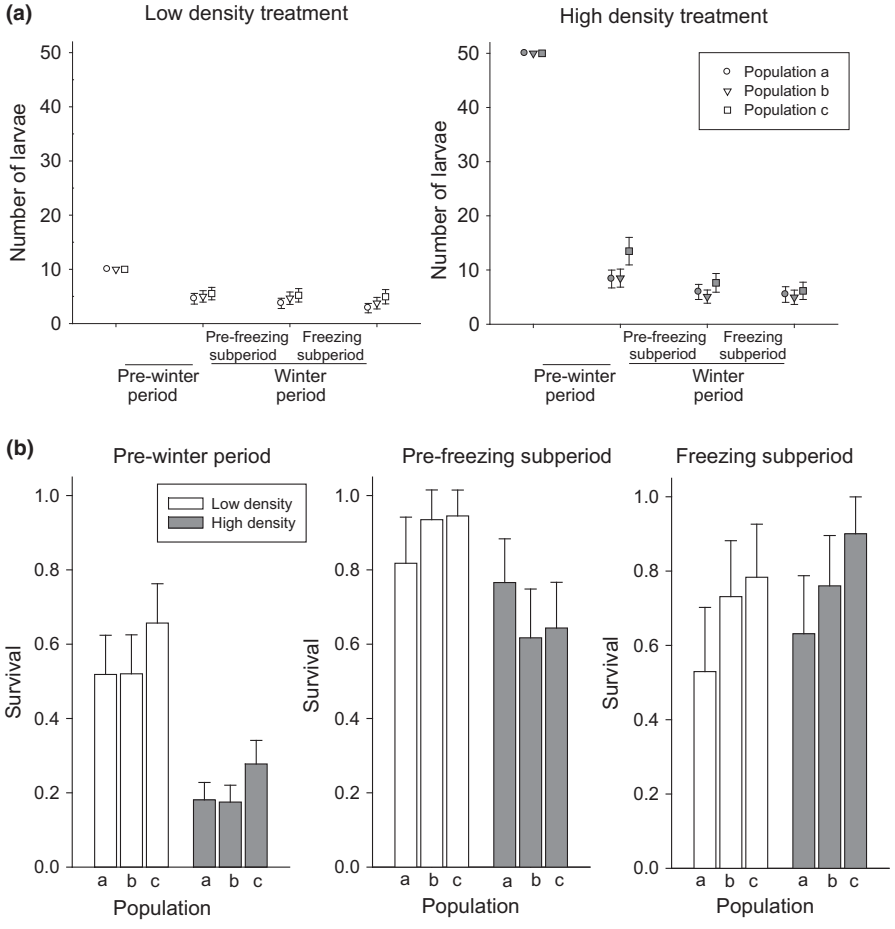

Fig. 3 (a) Mean number of *Coenagrion scitulum* larvae alive per mesocosm at the start of the pre-winter period, the end of the pre-freezing winter subperiod and the end of the freezing winter subperiod for the low- and high-density treatments. (b) Mean larval survival at the low- and high-density treatments during the pre-winter period, the pre-freezing winter subperiod and the freezing winter subperiod. Shown are least-square means with 1 SE. The initial larval density per mesocosm at the start of the experiment in summer was 10 larvae at the low-density treatment and 50 larvae at the high-density treatment. Means are shown separately for the three study populations.

Table 2 ANOVAs testing for the effect of the density treatment on (a) head width at the end of the pre-winter period and at the end of the winter period and (b) growth rate during the pre-winter period and winter period in larvae of *Coenagrion scitulum*. For head width, we tested also the effect of survival status (larvae that survived or were frozen to death) at the end of the winter period.

	Pre-winter period			Winter period		
	d.f.	*F*	*P*	d.f.	*F*	*P*
(a) Head width at the end						
Density treatment	1,25.5	82.91	<0.001	1,161	46.71	<0.001
Survival status				1,161	0.01	0.978
Density treatment × Survival status				1,161	2.41	0.123
(b) Growth rate						
Density treatment	1,24.7	66.8	<0.001	1,29	0.39	0.539

grew during the winter period and growth rate during the winter period did not differ between density treatments (Table 2b, Fig. 4b).

At the end of the pre-winter period, foraging activity of larvae was higher at the high-density treatment compared with the low-density treatment (Density treatment: $F_{1,49.7} = 11.9$, $P = 0.001$; Fig. 5a). Larger larvae had higher foraging activities (Head width: slope ± 1 SE: 0.304 ± 0.096, $F_{1,151} = 9.96$, $P = 0.002$).

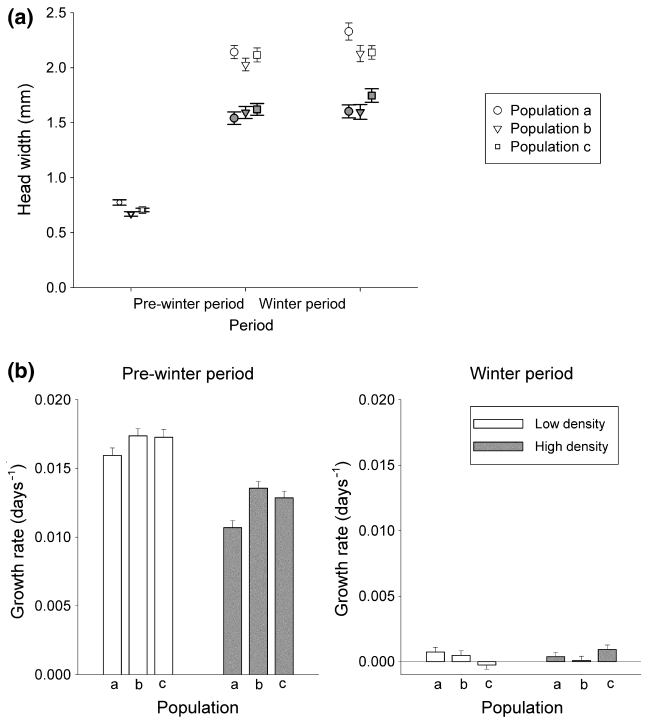

Fig. 4 (a) Mean head width of *Coenagrion scitulum* larvae at the low- (open symbols) and high-density treatment (grey symbols) at the start of the experiment, the end of the pre-winter period and the end of the winter period. (b) Mean growth rate at the low- and the high-density treatments during the pre-winter period and during the winter period. Shown are least-square means with 1 SE. Means are shown separately for the three study populations.

At the end of the winter period, final body mass of larvae was higher at the low-density treatment (Table 3, Fig. 5b). Final body mass was not dependent on the final density of larvae in the cage at the end of the winter (Table 3). The three condition estimates responded differentially to the density treatments. Larvae reared at high density had a lower size-corrected body mass, yet a higher fat content and a higher PO activity (Table 3, Fig. 5c–e). Furthermore, size-corrected body mass was lower in mesocosms with a higher final density (Final density, slope ± SE: −2.01 ± 0.68), while fat content and PO activity were not dependent on the final larval density at the end of the winter period (Table 3).

Discussion

The low-density treatment mimicking the low population densities at newly founded populations at the expansion front largely increased larval survival, growth rate and body size. The lower foraging activity at the low-density treatment indicates these positive effects on life history to work through a higher access to food at low conspecific density. The density treatment had diverse effects on the condition estimates, while size-corrected body mass was higher, fat content and PO activity were lower at the low-density treatment. The increased mortality at the high-density treatment equalised densities between density treatments at the end of the pre-freezing winter subperiod. Interestingly, initial density treatment had a larger effect than density experienced at the time of quantification on survival during the pre-freezing winter period and body condition estimates at the end of the winter, indicating delayed effects of the density treatment. Implications of the documented strong density dependence of traits in the larval stage in the scope of range expansion are further discussed.

During the pre-winter growth period, we documented strong effects of the high conspecific density on larval

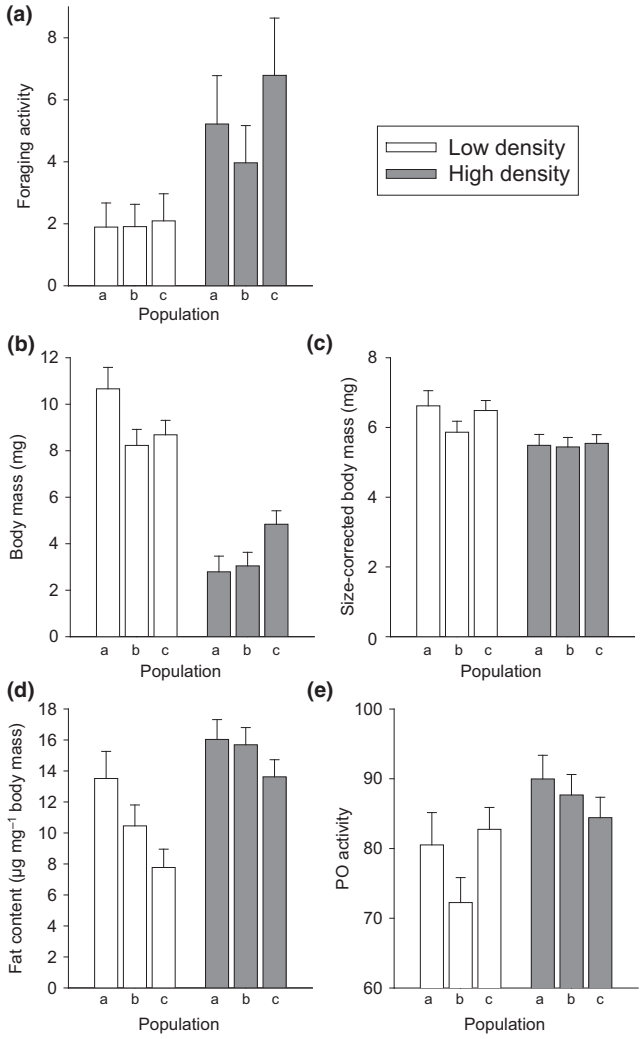

Fig. 5 (a) Mean foraging activity of *Coenagrion scitulum* larvae at the low- and high-density treatments at the end of the pre-winter period (a). Mean body mass (b), size-corrected body mass (c), fat content (d) and PO activity (e) at the low- and high-density treatments at the end of the winter period. Shown are least-square means with 1 SE. Means are shown separately for the three study populations.

survival, growth rate and size reflecting intense competition. This is in accordance with field studies indicating strong intraspecific competition among larval coenagrionid damselflies (Banks & Thompson, 1987; Anholt, 1990a). This competition may take the form of exploitation and interference competition leading to a decreased access to food resources (McPeek & Crowley, 1987; Anholt, 1990a). Many species, including *C. scitulum* (L. Therry, unpublished data), increase foraging activity under low food conditions to avoid too strong reductions in resource acquisition when food is limited (reviewed in Werner & Anholt, 1993; Anholt, Werner & Skelly, 2000). The observed lower foraging activity at lower larval densities thus suggests higher access to food at the low-density treatment and is supported by the observed higher growth rate and body condition (size-corrected body mass) at the low-density treatment. Such nutritional benefits at low population densities has been translated in high body condition at the expansion front of several species (Raby, Gutowsky & Fox, 2010; Brown, Kelehear & Shine, 2013) and has been suggested to imply a selective advantage for colonising patches beyond the expansion front and for accelerated range expansion (Brown *et al.*, 2013). While low population density may be a common feature of stationary and expanding range edges, the underlying reason for decreased conspecific density towards the range edge may differ fundamentally. Population density at stationary range edges decreases through limiting abiotic and biotic conditions (Hengeveld & Haeck, 1982; but see Sagarin, Gaines & Gaylord, 2006; Gaston, 2009) where low population density is in some cases accompanied by low body condition (Busch *et al.*, 2011). In contrast, initial low population density at expanding range edges may merely be shaped by the low number of individuals colonising patches beyond the expansion front (Burton & Travis, 2008).

Despite the equalised conspecific densities between density treatments after the pre-freezing winter subperiod, the density treatment strongly shaped body size and body condition at the end of the winter which has the potential to influence population dynamics at the expansion front. Survival of larvae during the pre-freezing winter period was lower at the high-density treatment. Interestingly, survival during the pre-freezing period was not influenced by the larval densities at the start of the pre-freezing period. This indicates delayed effects of the experienced high larval density during the larval growth season resulting in a lower ability to survive winter conditions.

The strong density-dependent mortality during the pre-winter period and during the pre-freezing subperiod equalised the final densities between the high- and low-density treatments at the end of the experiment, indicating the carrying capacity was reached in the mesocosms. Most species exhibit positive density-dependent dispersal (Matthysen, 2005), hence the equalised larval densities between density treatments may indicate equal dispersal propensities of the offspring between established populations and newly colonised edge populations. However, this conclusion is likely invalid for several reasons. Firstly, density dependence may evolve during range expansion. Simulations indicate that the rate of range expansion is highest under negative

Table 3 ANCOVAs testing for the effects of the density treatment (low versus high) and final density on body mass, size-corrected body mass, fat content and PO activity at the end of the winter period. Foraging activity and size-corrected body mass were corrected for larval head width.

	Body mass			Size-corrected body mass			Fat content			PO		
	d.f.	*F*	*P*	d.f.	*F*	*P*	d.f.	*F*	*P*	d.f.	*F*	*P*
Density treatment	1,131	89.28	<0.001	1,130	6.79	0.010	1,130	22.93	<0.001	1,130	8.88	0.003
Current density	1,26.7	1.59	0.219	1,82	8.80	0.004	1,59.2	0.50	0.481	1,74.3	0.49	0.488
Head width				1,129	613.77	<0.001						

density-dependent dispersal (Altwegg *et al*., 2013), and negative density-dependent dispersal may evolve during range expansion (Travis *et al*., 2009). Secondly, even with equal larval population sizes, effective adult population size is expected to be lower in high-density established edge populations. This is because besides direct lethal effects, the high-density treatment also likely has latent negative fitness effects. The observed smaller size and mass at the end of the winter period in the high-density treatment suggest that larvae were not able to compensate the lower body size by higher growth during the winter (De Block, Mcpeek & Stoks, 2007). This may have important fitness consequences as some slow growing larvae at high density will likely be unable to complete development in one year, which would result in a costly shift from a univoltine to a semivoltine life cycle (Banks & Thompson, 1987; Corbet *et al*., 2006). In case such voltinism shift takes place in established populations close to the expansion front, densities of dispersive propagules (adults) will decrease dramatically as fewer adults emerge from the larval pond in a given year when univoltine and semivoltine populations have equal larval carrying capacities. Additionally, larval mortality may be higher in semivoltine populations through stronger competition and cannibalism by older age cohorts (Crowley *et al*., 1987; Anholt, 1994; Purse & Thompson, 2002) and increased predation risk during the extended larval period (Berger *et al*., 2006). Alternatively, some animals may be able to avoid a voltinism shift by emerging at a smaller adult size (Blanckenhorn & Demont, 2004) whereby smaller adults are expected to have lower disperser abilities (Anholt, 1990b) and a reduced fecundity (Stoks & Cordoba-Aguilar, 2012). Furthermore, nutritional stress in the larval stage has been shown to decrease relative investment in flight muscles in the adult stage of the here studied species (Therry *et al*., 2014b), indicating that dispersal ability may be negatively affected through nutritional stress at the high initial larval densities of established populations.

The finding that two estimates of body condition (fat content and PO activity) were higher at the high-density treatment at the end of the winter period is unexpected but may reflect optimal investment in energy storage under food shortage. The higher fat content at high larval densities contrasts our expectation of lower food access, hence lower energy storage under larval crowding (e.g. Stoks, De Block & McPeek, 2006). Instead, our results are in agreement with the starvation–predation risk trade-off, which predicts that optimal energy storage should increase when food availability is lower (Higginson, Mcnamara & Houston, 2012) as expected at high population densities. Similarly, PO activity – a trait reflecting body condition (González-Santoyo & Córdoba-Aguilar, 2012) – was higher at the high-density treatment after winter. Additionally, larvae may have upregulated immune function to anticipate wounding and the associated increase of potential pathogen invasions at higher densities (Joop & Rolff, 2004).

Larvae were exposed to harsh winter conditions in the floating containers in which larvae were impeded to escape freezing conditions by moving deeper into the water column. Hence, the experienced winter conditions were even more extreme compared with those found in natural ponds located at the expansion front. Despite this, most larvae survived the freezing period, although they were fully encapsulated in the ice for 14 days. Canadian *Coenagrion* larvae often overwinter encapsulated in the ice (Sawchyn & Gillott, 1975), however, survival of a Mediterranean *Coenagrion* species in ice is surprising. The survival of the larvae under extreme winter conditions suggests that extreme winters have little impact on the distribution of *C. scitulum*. This is in line with Lingenfelder (2011) who observed no population declines and even the colonisation of new patches beyond the species' expansion front in Germany during two subsequent harsh winters. In contrast, in other poleward range-expanding species, winter survival is thought to be a major factor limiting the range expansion (Crozier, 2003; Roy-Dufresne *et al*., 2013; Lynch *et al*., 2014; Taulman & Robbins, 2014).

Overall, low densities at the expansion front seem to reinforce range expansion of the studied species. Effects

of the low population density at the range expansion front may differ between species and likely depend on species-specific density-dependent responses, whereby positive density dependence can slow down range expansion (Kot, Lewis & Van Den Driessche, 1996; Keitt, Lewis & Holt, 2001). Theoretical models exploring range expansion have demonstrated a strong impact of density regulation on the ability of tracking a shifting climate and the evolution of habitat specialisation during range expansion (Best *et al*., 2007; Filin *et al*., 2008). The expansion front is characterised by a steep gradient of decreasing population densities towards the expansion front (Phillips *et al*., 2010a). Direct effects of the density gradient across the expansion front are ignored in theoretical work simulating range expansions, while this density gradient may have an important impact on population dynamics. The here imposed low-density treatment typical for newly founded front populations strongly increased larval survival, growth rate and had long-lasting effects on body size and body condition. Furthermore, higher food accessibility in the larval stage may directly increase dispersal ability through a positive effect on flight-related traits (Therry *et al*., 2014b). A future challenge will therefore be to study how the higher post-winter body size and body condition at low initial larval densities at very edge of the expansion front influences spatial population dynamics, which significantly influence evolutionary trajectories in expanding populations (Hastings *et al*., 2005; Thuiller *et al*., 2008; McInerny *et al*., 2009).

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